# Bioprospecting of *Piper longum* L. endophytes for piperlongumine/ bioactive compound production, and effect of piperlongumine on beta-amyloid aggregation in *Caenorhabditis elegans* CL4176

Synopsis

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## INTRODUCTION

Endophytes are the microbes which include fungi, bacteria, and actinomycetes that reside within the host plant for a part of their lifecycle or whole without causing any harmful effects to the plant or their tissues. (Petrini and Fisher, 1990; Arnold, 2007). Endophytes are responsible for the production of a wide range of medicinal or bioactive compounds in plants which make them quite useful in pharmaceutical industries. Some of the bioactive compounds may include flavonoids, alkaloids, phenolic acids, benzopyranones, terpenoids, steroids, tetralones, quinones etc. (Tan and Zou, 2001).

Fungal endophytes are capable of producing many important secondary metabolites which are required in the production of many natural products. Such natural products have wide ranges of application in medicine, agriculture, and pharmaceutical industries (Strobel and Daisy, 2003; Selim *et al*., 2012). Moreover, endophytic actinobacteria are also responsible for the production of many significant natural products (Ryan *et al*., 2008; Qin *et al*., 2011). They are mainly known for their ability to produce many secondary metabolites which imparts antitumor, antibiotic and immunosuppressive properties to the plants. Therefore, exploring the properties of such actinobacteria has many significant applications (Locci., 1989). There have been many reports regarding the beneficial properties of endophytes isolated from various plant species but fungal endophytes and actinobacteria living in symbiotic association with *Piper longum* are least explored. As *P. longum* is an important spice with many medicinal properties, endophytes isolated from this plant are expected to be good source of secondary metabolites having diverse bioactive compounds which have significant applications in drug industry.

Alkaloids are defined as heterocyclic natural compounds with one or more nitrogen atoms which have diverse clinical properties. The term alkaloid has been derived from the basic nature of many members of this group. However, many nitrogen-containing molecules do not necessarily belong to this group. Alkaloids have limited distribution in the plant kingdom. Roots of *P. longum* plant are the rich source of the amide alkaloid Piperlongumine (PL), which have many useful medicinal properties. Piperlongumine possess many significant properties like anticancerous, antidiabetic, anti-platelet aggregation, and antifungal activities. Therefore, endophytic fungi and actinomycetes isolated from *P. longum* are expected to be the rich source various useful alkaloids.

PL has been known to possess various pharmacological effects in a wide number of conditions like neurodegenerative disease, psychiatric disorders, tumours, diabetes and pain (Go *et al.,* 2018). Alzheimer is a neurodegenerative human disease, characterized clinically by a progressive and rapid decline in cognitive function and neuropathologically by the presence of neuropil threads. It is also accompanied by specific neuron loss, and synapse loss, in addition to the hallmark findings of neurofibriallary tangles and senile plaques (Lee *et al.,* 2001). The nematode worm *Caenorhabditis elegans* has been used as model organisms in many recent literatures in order to study and analyze the details of Alzheimer.

**REVIEW OF LITERATURE**

Endophytes can be defined as microorganisms such as fungi, bacteria including actinomycetes which expend either the complete or part of their lifecycle within the healthy tissues of a living plant, typically causing no symptoms of disease (Gunatilaka *et al.,* 2006). In 1993 it was first reported that endophyte, *Taxomyces andreanae* produces the same bioactive secondary metabolite taxol (paclitaxel) as its host *Taxus brevifolia* (Stierle *et al*., 1993).

The importance of endophytes had been demonstrated over a long period as a source of pharmaceutical bioactive compounds, as many of the endophytes were exposed to produce novel bioactive metabolites such as antibacterial, antifungal, antiviral, antitumor, antioxidant, anti-inflammatory, immunosuppressive drugs, and many related compounds (Anjum & Chandra, 2015). Endophytes enter the plant tissue chiefly through the root zone (Kobayashi, 2000). Many plant processes have been shaped through association with endophytic fungi. For example, endophytic fungi are suggested to play a major role in structuring plant communities and in shaping processes such as colonization, competition, coexistence and soil nutrient dynamics (Saikkonen *et al*., 2002). Endophytic fungi are now recognized as sources of pharmacologically beneficial, novel bioactive compounds.

The discovery of endophytic fungi capable of producing the same bioactive compounds as their host medicinal plant has raised the expectation that these compounds could be produced in large scale through fermentation processes, thus meeting the growing demand of the market (Kumari *et al*, 2018). The production of novel bioactive compounds by endophytic actinobacteria isolated from a wide range of plants like tomato, banana, wheat, and maize with promising antipathogenic activities (Coombs & Franco, 2003; Castillo *et al*., 2007). It was also reported that an endophytic actinobacteria,

*Pseudonocardia*, isolated from *Artimisia annua* was found to enhance production of artemisinin (Li *et al.,* 2012).

Bioactive compounds produced by plants are mainly known as plant secondary metabolites. They are usually synthesized within the plants, besides their association with primary biosynthetic and metabolic routes for compounds associated with plant growth and development (Goyal, 2019). Many of them are responsible for several types of important activities in the living plants like protection, attraction or signaling (Bernhoft, 2010). Medicinal plants are the most promising source of different kinds of natural bioactive products and secondary metabolites producing endophytes which have the capability to attack particular proteins coded by essential genes (Kingston, 2001).

Piperlongumine is one of the promising alkaloids isolated from the medicinal plant *Piper longum*. Many *in vivo* and *in vitro* experiments have proved that PL possesses anti-inflammatory, anti-carcinogenic and anti-atherosclerotic activities. Moreover, it has also been employed in the treatment of gastrointestinal complaints, respiratory diseases and malaria (Liang *et al*., 2020). PL has gained increasing attention because of its anti-cancerous property. Its anti-cancerous actions include inhibition of the proliferation of various human cancer cell lines under *in vitro* conditions. It has also been reported that death rate of cancerous cells increases in the presence of PL (Wang *et al*., 2015; Lee *et al*., 2015).

The potential of many promising endophytes had been mostly exploited in the field of pharmaceutical and drug industries (Anjum & Chandra, 2015). Many major processes of plants like coexistence with other organisms or pathogens and adjustment to soil dynamics are mostly influenced by endophytic fungus (Saikkonen *et al*., 1998). As endophytic fungi are well known for their pharmacologically beneficial properties and for the production of useful bioactive compounds, many researchers have focused in large scale production of such compounds through fermentation processes in order to meet the increasing demand in pharmaceutical and drug industries (Kumari *et al*, 2018). Endophytic actinobacteria are also appreciated for their significant anti-pathogenic activities. (Coombs & Franco., 2003; Castillo *et al.,* 2007).

It is very important to hunt for alternative approaches to produce the medicinal plant-derived biologically active compounds, particularly for those which have been isolated from endangered or not easy growing plants in order to fulfill the increasing demand for medicines and drugs. This problem can be solved to

some extent by utilizing the natural products extracted from plant cell and tissue culture which are found in more quantity (Atanasof *et al.,* 2015; Rai *et al*., 2016; Ramirez-Estrada *et al*., 2016). The same can also be achieved by exploiting the endophytic fungus and actinobacteria residing within plants which are well known as rich source of various bioactive compounds. They are capable of producing similar or same secondary metabolites as their host plant (Zhao *et al.,* 2011). Actinomycetes are prokaryotic microorganisms that belong to the phylum Actinobacteria and possess mycelium like fungus and forms spores (Chaudhary *et al*., 2013; Barka *et al.,* 2016). Several literatures have reported that that secondary metabolites found in plants are also produced by endophytes (Zhao *et al.,* 2011). Many important plant processes are influenced by their symbiotic association with fungi.

*Caenorhabditis elegans* has been progressively being used as a model organism for the study of neurodegenerative diseases. As nematodes are translucent, they can be comfortably utilized in the study of particular neurons in living animal, and easy to manipulate genetically. Inspite of the vast evolutionary divergence, human proteins are functionally active when expressed in *C. elegans*, and disease-associated mutations in these proteins also result in phenotypic changes in the nematode. Wolozin *et al,* (2011) reported the use of *C. elegans* to analyze the pathophysiology of Alzheimer's disease, Parkinson's disease, and axonal degeneration. The particular study has provided that strong phenotypic effects were seen in *C. elegans,* and in many cases reproduce selective neuronal vulnerability observed in humans (Wolozin *et al*., 2011).

**OBJECTIVES**

The present study is targeted on the isolation of endophytic fungi and actinobacteria from *Piper longum* L. which have promising role in the production of useful alkaloids like Piperlongumine and to study the effect of Piperlongumine on *C. elegans. P. longum* is an important medicinal plant of India, which belongs to the family Piperaceae. It is also known as long pepper in English and Pipali in Hindi. *P. longum* is a flowering creeper and mainly for its spike which is dried and used for seasoning and medicinal purposes**.** *P. longum* is commonly used to treat diseases like malaria, bronchitis, paralysis of tongue, constipation, asthma, chronic malaria etc. It is also the rich source of many bioactive compounds like alkaloid Piperine and Piperlongumine etc. (Wiemann, 2017).

The proposed study will focus on following objectives: -

1. Collection of root samples from different accessions of *P. longum*.
2. Isolation and identification of fungal and actinobacterial endophytes.
3. Preparation of crude extract from the isolated endophytes.
4. Effect of isolated crude extract and standard Piperlongumine on β- amyloid aggregation in *C. elegans*.

**METHODOLOGY**

Endophytes will be isolated from roots of healthy plants of *P. longum* belonging to various accessions and procured from different geographical regions of India. Collected plant samples will be air-dried followed by through washing to remove dirt and soil present on the external surface of the plant.

**Surface sterilization**

For surface sterilization, plant samples will be treated with 70% ethanol for 1 minute, 3 to 5% sodium hypochlorite (3 to 5% chlorine) for 5 minutes and then with 96% ethanol for 30 seconds. Finally, the sample will be rinsed with sterile distilled water for several times. Proper surface sterilization of the plant material shall be validated through the following steps: (i) imprints of sterilized plant parts should be taken on 1 Petri dish containing sterile culture media and (ii) 0.1 ml of last rinsed water will be spread on nutrient media. If no microbial growth is visible on these Control plates for 4-7 days of incubation at 26 ºC to 28 ºC, then the sterilization procedure will be considered effective (Verma *et al.*, 2011; Phurailatpam, 2019).

**Isolation of fungal and actinobacterial endophytes**

Roots will be separated from the plant material and subjected to proper sterilization protocol. Sterilized plant material will be cut into segments of approximately 0.5-1cm and inoculated on suitable plant minimal media at ~27 ℃ to 30 ℃ for about 4 weeks. The nutrient media for fungal endophytes will be potato dextrose agar, malt extract agar and water agar etc. Different isolation media of actinobacteria will be Yeast Extract-Casein hydrolysate Agar, Starch Casein Agar, Humic acid vitamin B Agar. Isolated fungal and actinobacteria endophytes will be purified and maintained in appropriate medium for further analysis (Verma *et al.*, 2011).

**Preparation of crude extract from endophytic culture**

Different isolates will be inoculated in different suitable broth media for the preparation of liquid culture. The inoculated broth media will be kept in a shaking incubator from 7 days to one month at appropriate temperatures for the optimization of alkaloids production. After incubation the culture will subjected to centrifugation at 10000 rpm for 10 minutes to obtain the culture supernatant. The supernatant will be acidified to pH 2 with 1N HCl if required. The acidified supernatant will be dissolved in equal volume of ethyl acetate and kept in a shaker for 30 minutes in order to dissolve the alkaloids present. This step will be repeated for 2 to 3 times depending on the requirement and finally the solvent phase will be collected. The solvent phase will be allowed to evaporate at 45 ℃ under vacuum in a rotatory evaporator or oven. The dry residue collected after evaporation will be weighted and re-suspended in 1.5 ml of methanol and stored in refrigerator for further analysis (Kumar *et al*., 2013). Un-inoculated or blank broth undergoing exactly the above-mentioned procedure will serve as negative control.

**Preliminary screening for the presence of alkaloids**

For the test of alkaloids, 2ml of each extract will be acidified with a few drops of dilute hydrochloric acid. Then 1ml of Dragendorff's reagent will be added. The presence of red- orange colour indicates that the alkaloids are present in the precipitate (Thilagavathi.T *et al*., 2015).

**Qualitative and quantitative analysis of crude extract for the presence of bioactive compounds**

Qualitative analysis for the presence of any bioactive compounds will be done through chromatographic technique, TLC method. Quantitative analysis will be carried out through HPTLC technique. The HPTLC method will be done according to the protocol of Pundarikakshudu *et al*. (2014) by using hexane–ethyl acetate (4:1, v/v) as solvent system. The absorbance was measured at 325 nm using a UV-Visible spectrophotometer. The detection will be performed at corresponding wavelength for Piperlongumine (Pundarikakshudu *et al*., 2014).

**Molecular identification of isolated endophytes**

The isolated endophytes will be identified using specific RNA sequencing technique. For actinobacteria 16S rRNA sequencing will be used and for fungi ITS sequencing will be used or any other similar method. Genomic DNA will be isolated from promising endophytes in order to use as template for PCR

using appropriate forward and reverse primers. The amplicon obtained will be used for sequencing and identification through BLAST tool of NCBI.

**Culture of *C. elegans***

The worms will be cultured in nematode growth medium (NGM) supplemented with E. coli OP50 as a food source and will be kept in an incubator at 20 °C (Brenner,1974).

**Age synchronization of *C. elegans***

Age synchronization of the worms will be done by isolating eggs from gravid hermaphrodites. The eggs will be prepared through addition of lysis solution containing 5 M NaOH and 5% NaOCl, followed by vortexing for 10 min. After centrifugation for 2 min at 1800 rpm, supernatant will be removed, and the pellet obtained will be washed once in distilled water before centrifugation of another 2 min. After centrifugation, water will be discarded and the remaining eggs will be resuspended in suitable buffer for hatching.

**Treatment of *C. elegans* with crude extract**

To analyze the effect of isolated crude extract in *C. elegans,* the medium will be supplemented with different concentration of crude extract of endophytes isolated from *P. longum*. Both the strains of E. coli OP50 and β- amyloid strains of *C. elegans* that will be used in the proposed study will be procured from Caenorhabditis Genetics Center (CGC), University of Minnesota, USA. All the experiments will be conducted in young adult worms having similar age group (Prashanth *et al*., 2019).

**β- Amyloid aggregation**

*C. elegans* CL4176 will be used for the study of the effect of crude extract. Young adult worms will be selected randomly and incubate them in 25°C incubator for 24 h to induce human Aβ expression. Aggregation of β– Amyloid will be measured by microplate reader (Xu *et al*., 2019).

**IMPORTANCE OF STUDY**

The present study has been designed to find an alternative source of Piperlongumine or other bioactive compounds, which are otherwise obtained from the roots. Destructive harvesting of roots poses a major risk for the biodiversity. In addition, the effect of bioactive compounds including PL, will be determined on the β-Amyloid aggregation, a characteristic feature of Alzheimer's disease. Alzheimer's disease does not have any proper treatment till today. Many of the medicines prescribed for this disease are only

meant for reducing the symptoms. If PL shows significant effect on the neurodegenerative disease in *C.elegans*, then it will be a promising target for application the pharmaceutical and drug industry.

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